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Chromatographic Purification and Functional Properties of Cytochrome *c* Oxidase

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CHROMATOGRAPHIC PURIFICATION AND FUNCTIONAL PROPERTIES OF CYTOCHROME *c* OXIDASE

by

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Avhandling för filosofie doktorsexamen i kemi (examinator professor Bo G Malmström), som med kemiska sektionens vid Göteborgs universitet medgivande kommer att offentligt **försvaras** fredagen den 31 mars 1978 kl 10.15 i hörsal KA, **Kemihuset**, Chalmers tekniska högskola, Göteborg.

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This thesis is presented as a summary based on the following papers:

I G. Lang, S. Lippard and S. Rosén: Mössbauer Spectroscopy Studies of Beef-Heart Cytochrome *c* Oxidase. (1974) Biochim. Biophys. Acta 336, 6-14

II S. Rosén, R. Brändén, T. Vänngård and B.G. Malmström: EPR Evidence for an Active Form of Cytochrome *c* Oxidase Different from the Resting Enzyme. (1977) FEBS Lett. 74, 25-30

III S. Rosén: Purification of Beef-Heart Cytochrome *c* Oxidase by Hydrophobic Interaction Chromatography on Octyl-Sepharose CL-4B. (1978) Biochim. Biophys. Acta, in press

IV S. Rosén: Kinetic Studies on Oxidized and Partially Reduced Cytochrome *c* Oxidase. (1978) Biochim. Biophys. Acta, in press

INTRODUCTION

Life is a stationary state which is maintained by a controlled flow of matter and energy. Most of the energy required for reactions in living systems is derived from the hydrolysis of adenosine triphosphate, ATP. A large part of this substance is synthesized by utilizing chemical energy released during oxidation of reduced, energy-rich metabolites from the degraded food. This process is called oxidative phosphorylation. The oxidation-reductions take place in the respiratory chain, a membrane-bound system of proteins that is found either in mitochondria, which are the respiratory organelles of highly developed cells, or in the cytoplasmic membrane, as is the case with bacteria.

In aerobic organisms, oxygen is the ultimate electron acceptor in the consecutive series of oxidative reactions. Dioxygen is a poor oxidizing agent with most organic substances, but certain enzymes, called oxidases, increase its rate of reduction. Only a few oxidases, however, such as cytochrome *c* oxidase, laccase and ascorbate oxidase utilize the full oxidizing capacity of oxygen. In this process oxygen is reduced all the way to water, past the levels of hyperoxide radical, $HO_2^{\cdot}$, peroxide, H_2O_2 and hydroxyl radical, $OH^{\cdot}$.

Cytochrome *c* oxidase is the most important of these enzymes. This protein is the terminal component of the

respiratory chain, and it is estimated to be responsible for about 90% of the total oxygen consumption of living organisms. Thus, its general biological significance exceeds that of hemoglobin, which merely acts as an oxygen-transporting protein in those higher organisms, where diffusion of oxygen is insufficient to satisfy the needs for this oxidant.

The protein components of the respiratory chain are flavoproteins, iron-sulfur proteins and cytochromes, and the latter have the strongest absorption bands in the visible region (400-700 nm) of the electromagnetic spectrum. Four absorption bands were detected by Mac-Munn as early as 1886 [1], but all the bands were considered to originate from a single protein. It was not until 1925 that the four-banded spectrum was given a satisfactory explanation when Keilin [2] concluded that three separate cytochromes, *a*, *b* and *c*, were associated with the spectrum. Keilin thought, however, that the terminal oxidase, at that time named indophenol oxidase, was a copper protein, containing no heme iron. This point of view was strongly rejected by Warburg, who showed that the terminal component, by him called "Atmungsferment", in fact was a hemoprotein [3]. A long-lasting controversy followed, but in 1939 the conflict was finally resolved when it became clear, that the substance previously named cytochrome *a* contained more than one heme, the second one behaving like Warburg's Atmungsferment [4]. This component was called cytochrome a_3 to distinguish it from cytochromes *a*, a_1 and a_2; the last

two symbols had in the meantime been assigned to respiratory components in certain bacteria. In spite of much effort, a separation of two active cytochromes has never been made, and therefore the term cytochrome aa_3 for the terminal enzyme has generally been replaced by the name cytochrome oxidase.

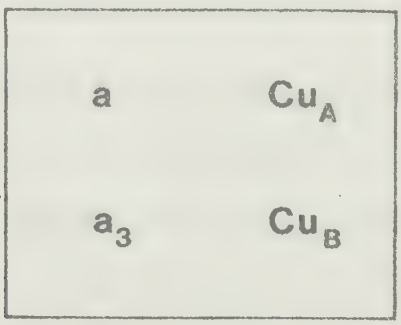


Fig. 1 Functional unit of cytochrome oxidase

The minimal functional unit of cytochrome oxidase contains two heme iron and two copper ions (Fig. 1), and from the heme-to-protein ratio as well as from amino acid analysises its molecular weight has been calculated to about 200.000 (see ref. 5 for a review). The actual molecular weight of this unit is, however, about 240.000, the difference being caused by bound phospholipids, mainly phosphatidylcholine and phosphatidylethanolamine. The enzyme obtained both from beef heart and from yeast contains seven distinct subunits, ranging in molecular weights from 4.500 to 40.000 [6-8], but the number of the polypeptide chains and their arrangement is not yet established. Three subunits with a high hydrophobicity are synthesized by mitochondrial DNA, whereas the remaining four subunits have nuclear origin [9,10]. A similar situation is

also found with another protein in the mitochondrial inner membrane, adenosine triphosphatase (ATPase) [11]. The mitochondrial protein synthesis is blocked by agents which specifically inhibit protein synthesis in bacteria, and it has been suggested possible that mitochondria once originated from bacteria which have fused with other cells [12].

Heme groups are in general sensitive probes of pertubations in their environment, illustrated by changes in their redox potentials and absorption spectra and by spin state transitions [13]. Much of the work in this thesis therefore deals with studies on the hemes of cytochrome oxidase in an effort to elucidate structural and functional properties of the enzyme.

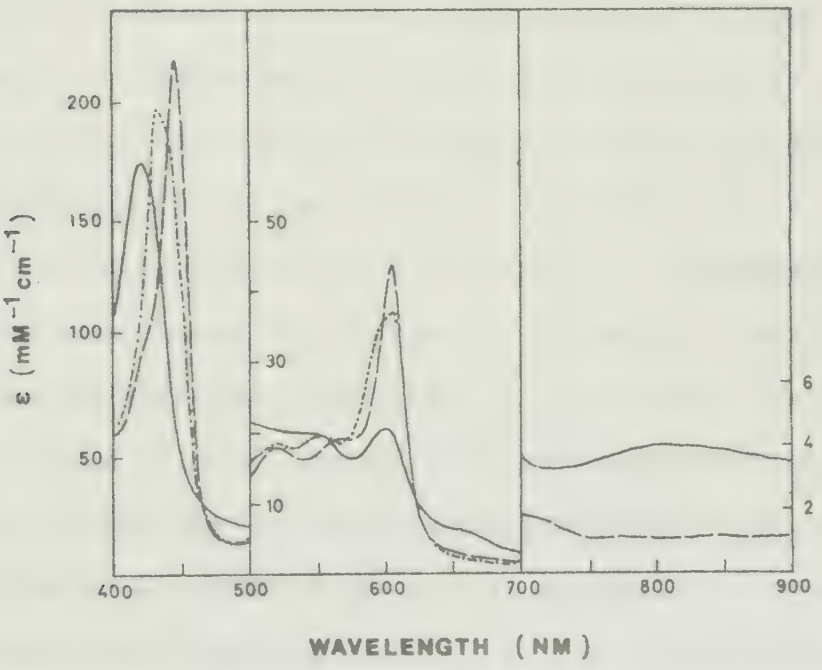


Fig. 2 Optical absorbance spectra of oxidized (———), reduced (- - -) and CO-cytochrome oxidase (-·-·-) at 25°C. The CO-spectrum was not recorded in the near-infrared region.

A new purification procedure for cytochrome oxidase

Most of the preparation methods for cytochrome oxidase that have been published (see 5 for a review) do not differ in principle from the original procedure of Straub [14] and of Yahushiji and Okunuki [15]. The anionic detergent from bile, sodium cholate, is utilized for solubilization of the mitochondrial membrane, and ammonium sulfate fractionations are used for the separation of cytochrome *b*, c_1 and *c* from cytochrome oxidase. Methods based on column chromatography have a higher resolving power, but ion-exchange chromatography has not been successfully applied to cytochrome oxidase from beef heart. The protein has been purified by affinity chromatography using Sepharose®-bound cytochrome *c* [16], but the results could not be reproduced in our laboratory. Experiments showed, however, that when cytochrome *c* was bound to Sepharose 6 B via a diglycidyl ether and equilibration was made at low pH and low ionic strength (10 mM phosphate buffer, pH 6.0, containing 0.5% of the non-ionic detergent Tween 80), part of the oxidase was bound to the column (B. Bejke, I. Olsson, J. Porath and S. Rosén, unpublished results). In this case the binding was probably in part caused by electrostatic interactions, since the difference in isoelectric pH for the two proteins is about 5 pH units, cytochrome *c* being the more basic one with pI = 10.6. As only 30-50% of the enzyme was adsorbed, the method was not applied on a preparative scale, but the results are interesting and deserve further study.

In recent years hydrophobic interaction chromatography has been successfully utilized for the purification of many water soluble proteins, which bind to alkyl and aryl derivatives of agarose at high salt concentrations [17-19]. Desorption of the proteins can be accomplished by a decrease in the salt concentration [18] or by the inclusion of ethylene glycol [17] or detergents [20] in the eluting buffer. As detergents are necessary for solubilization of membrane components, one might expect that the proteins in the extract would not bind to the hydrophobic gels. Despite this, cytochrome oxidase was found to bind strongly to Octyl-Sepharose CL-4B in the presence of cholate, and paper III describes a purification based upon this finding. It is shown that under these conditions most of the cytochromes *b* and c_1 are slightly retarded only and thus can be washed out of the column. By replacing cholate with the non-ionic detergent Triton X-100 (*t*-octylphenoxypolyethyleneglycol), the enzyme is eluted in a purified form. The procedure is simple, involving only one ammonium sulfate precipitation before application to the column, without any intermediary gel filtration or dialysis.

Characterization of the enzyme with electron paramagnetic resonance (EPR) and optical spectroscopy shows that the usual criteria of a good preparation are satisfied. Polyacrylamide gel electrophoresis performed under highly dissociating conditions indicates that the smallest subunit is missing in the purified

enzyme. This finding casts doubts on a recent claim that this subunit contains one of the two functional copper ions [21]. It should be mentioned, however, that the separation of the peptides was not as good as in experiments with conventionally prepared cytochrome oxidase, possibly because of the presence of Triton. The activity of the oxidase, after addition of phospholipid, is about the same as other preparations which means that if the smallest subunit is missing, it is not necessary for the electron-transport function of the enzyme.

The question of separate cytochromes a and a_3 in the resting enzyme

It was recognized already in the 1920s that only one cytochrome, nowadays designated cytochrome a_3, reacts with azide, cyanide and carbon monoxide [22]. This seemingly strong evidence for cytochrome oxidase being asymmetric was questioned when it became clear that only one type of heme was isolated after extraction of the enzyme with acetone and hydrochloric acid (Fig. 3).

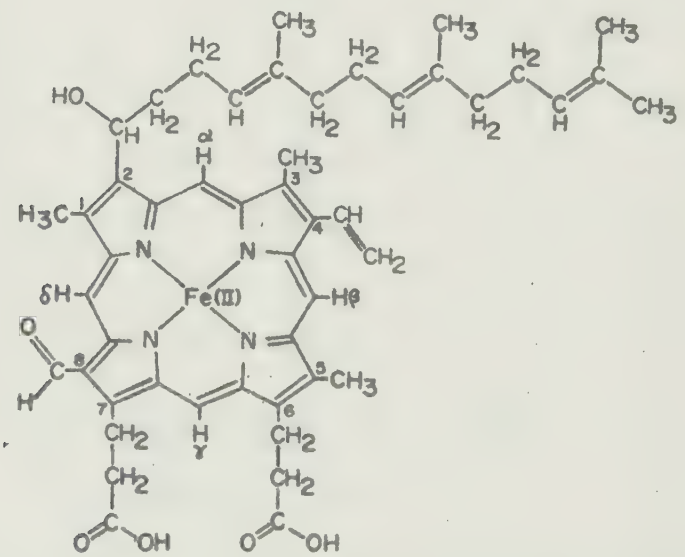


Fig. 3 Structure of heme A

It was instead suggested that the enzyme consists of a symmetric dimer of two identical protein molecules, and the reactivity towards various ligands was explained by negative cooperativity [23,24].

From reductive titration experiments it has been suggested that the metal ions exist as Fe(III) and Cu(II)

in the oxidized enzyme [25,26], and as these contain one or more unpaired electrons each, they should be paramagnetic. One would expect, then, that EPR spectroscopy should reveal four different components in the oxidized enzyme (see Fig. 1). Only one heme signal and a signal close to $g2$, generally ascribed to Cu^{2+} [27] is seen at 77 K, however (Fig. 4).

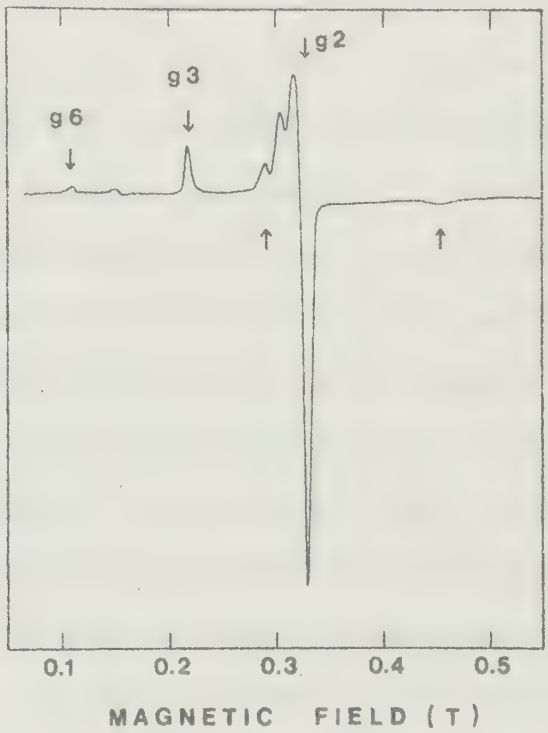


Fig. 4 EPR spectrum of oxidized cytochrome oxidase at 12 K, showing the low-field positions of high-spin heme ($g6$) and low-spin heme ($g3$) and the signal close to $g2$ which is associated with Cu_A. The other two arrows show absorption lines derived from the same low-spin heme which gives the $g3$ line.

The intensity of the $g2$ signal corresponds only to about half of the total copper content, and the signal has been assigned to one of the two copper ions, here denoted Cu_A. The reason for the absence of a signal from the

other copper ion, Cu_B, will be discussed later in this section. The heme signal shows properties typical of a low-spin heme, e.g. as in cytochrome *c*. At 77 K a correct estimation of the intensity was difficult, so one could not decide whether the signal represented one or both hemes.

Mössbauer spectroscopy is different in principle from EPR, as the ions or atoms investigated do not have to be paramagnetic. The sample is irradiated with highly monochromatic γ-rays, the energy of which can be shifted by means of the Doppler effect, i.e. by oscillating the γ-source at moderate velocities. The resonant absorption of the γ-rays by the nuclei in the sample yields information on the *s*-electron density and on the electric and magnetic fields at the nucleus. These factors are sensitive to the nature and geometric arrangements of the ligands. Only a few isotopes of biological significance are suitable, and among these is the stable isotope of iron, ^{57}Fe, which occurs naturally with an abundance of 2.2%, and consequently Mössbauer spectroscopy is applicable to heme proteins.

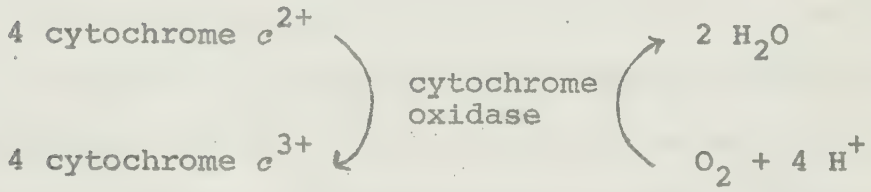
The results of the Mössbauer study in paper I were best explained by the two hemes not being identical in the resting, oxidized enzyme. One component had properties typical of a low-spin heme, and additional lines were tentatively interpreted to originate from a high-spin heme, with parameters close to those of the high-spin

heme in cytochrome *c* peroxidase. The signal-to-noise ratio was not very good because of the low abundance of ^{57}Fe, but since the hemes cannot be removed reversibly, enrichment in this isotope was not possible with enzyme from beef heart. Efforts have been made in this and other laboratories to prepare yeast cytochrome oxidase highly enriched in ^{57}Fe, but so far without success.

Later EPR studies at lower temperatures [28] have confirmed that the intensity of the low-spin ferric heme only corresponds to one heme iron, and it is suggested that the other iron is antiferromagnetically coupled to the EPR-undetectable copper, Cu_B [27], thereby explaining the lack of EPR signals from these ions. This hypothesis has received strong support from recent room temperature susceptibility measurements with an NMR technique [29].

Catalytic intermediates of cytochrome c oxidase

Apart from investigations on structural properties of the oxidase, which were dealt with in the last section, it is also necessary to study functional properties of the enzyme in order to obtain a knowledge of how electrons are transferred from ferrocytochrome *c* to oxygen:



The aim of papers II and IV is to give a better characterization of the partially reduced oxidase and to suggest enzyme intermediates relevant for the catalytic reaction. This process might in principle be simple, involving four separate one-electron transfers to oxygen, one from each of the four metal ions, which all are potential electron acceptors. From thermodynamic considerations such a mechanism is fairly unlikely, since the reduction of oxygen to hyperoxide has a reduction potential much lower than the acceptors in cytochrome oxidase (see [30]). The presence of an antiferromagnetic coupling between two of the metals in the oxidized enzyme [29] serves as an indication of a mechanism in which interaction among components is an essential feature, which would be expected in multi-electron transfer.

Many investigations on the oxidation-reduction properties of the enzyme have been performed, the knowledge of which is important in elucidating what molecular species or forms may be involved in catalysis. Experimental evidence shows that cytochrome oxidase behaves in a complex way. Thus, at least two forms of the enzyme exist where all the metal ions are fully oxidized, namely the resting enzyme which has an absorption maximum at 420 nm in the Soret region (400-450 nm), and the so called oxygenated enzyme which can be produced on reoxidation of partly or fully reduced enzyme, giving a maximum at 428 nm [31,32]. Reduction experiments with ferrocytochrome *c* in the absence of oxygen reveal another complexity, as the redox properties of the enzyme after the initial reduction of the primary acceptor, believed to be a heme, differ as compared to at equilibrium [33-35]. EPR investigation at 12 K also showed remarkable features. Thus, high-spin heme signals, characterized by a resonance at *g*6, appear at intermediate levels of reduction [36]. These signals are absent in the fully oxidized and fully reduced enzyme and therefore provide evidence of interaction between metal ions in the enzyme. Furthermore, the time-dependence of the signals may reflect the changes in redox properties mentioned above: after the initial reduction, a high-spin heme with a nearly axial symmetry is obtained (Fig. 5, lower trace), which under anaerobic conditions is slowly converted to a high-spin species with a lower symmetry, called rhombic high-spin heme [37] (Fig. 5, upper trace).

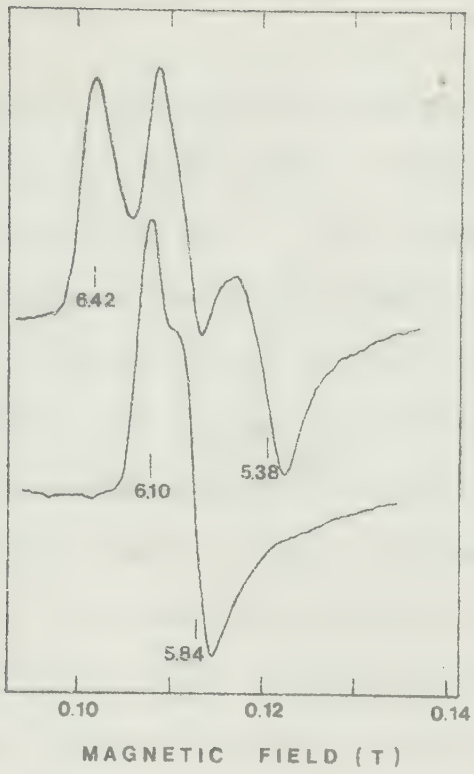


Fig. 5 High-spin heme signals at 12 K of cytochrome oxidase obtained 40 ms (lower trace) and 15 min (upper trace) after anaerobic mixing of cytochrome oxidase with one equivalent of ferrocytochrome *c* at room temperature. The numbers show *g*-values for the rhombic high-spin heme in the upper trace and for the component obtained after rapid reduction.

The intensity of the high-spin heme signals did not correspond to more than 0.3 hemes with ferrocytochrome *c* as reductant [28]. Experiments were also performed with both substrates, cytochrome *c* and oxygen, present in a search for spectral shifts in the visible region. For this purpose, a rapid scanning stopped-flow spectrophotometer was used, in which 200 nm is scanned in 1 ms. The results were, however, negative in the investigated time-interval 0.003 - 1 s (S. Rosén and M. Holloway, unpublished experiments).

In light of the fact that there exist at least 10 EPR signals of cytochrome oxidase [28] and with the above considerations in mind, it is no simple matter to determine which enzyme species are relevant for catalysis. In papers II and IV a new approach was used in the studies of the oxidase. The enzyme was first incubated anaerobically with 1-2 equivalents of ferrocytochrome *c* until equilibrium had been reached, whereafter the kinetic properties of the partially reduced enzyme were studied after rapid mixing with ferrocytochrome *c*, O_2 or CO. In some experiments the fully reduced enzyme was used. It was shown in paper II that the one-electron-reduced cytochrome oxidase rapidly oxidized ferrocytochrome *c* with a simultaneous decrease in the low-spin heme signal and rhombic high-spin heme signal of the oxidase. In paper IV these observations were confirmed and extended. Thus, the kinetics and extinction changes obtained during this reaction suggested involvement of the same acceptor as in the resting enzyme. The results imply an increase in the rate of intramolecular electron transport from the primary acceptor to other sites in the enzyme. In contrast to the one-electron-reduced oxidase the two--electron-reduced enzyme oxidized a very small amount of anaerobically added ferrocytochrome *c*.

It was also demonstrated that the one-electron--reduced enzyme reacted rapidly with both oxygen and carbon monoxide. In the reaction with oxygen, the rhombic

high-spin heme signal disappeared within 40 ms [37]. In paper II it was shown that though this signal is formed slowly in anaerobic reactions, it could be formed rapidly by mixing the fully reduced enzyme with ferrocytochrome *c* and a stoichiometric amount of oxygen. EPR experiments were also performed both with small excess of substrate in the presence of oxygen and at conditions which were close to steady-state (paper IV). Only the high-spin heme signal in Fig. 5, lower trace, was obtained during the course of oxidation of reduced cytochrome *c*.

The results demonstrate that at least some molecules, containing rhombic high-spin heme, react rapidly with both ferrocytochrome *c* and oxygen and therefore possess properties which are of relevance in catalysis. This finding, combined with the poor ability of two-electron--reduced enzyme to oxidize ferrocytochrome *c*, argues against a notion that the enzyme has to be fully reduced before it can participate in turnover [38]. If rhombic high-spin heme species are involved in catalysis, it also follows that one effect of oxygen must be to facilitate a rapid formation of these species.

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